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APPLICATION NUMBER: ***60/393,747***
FILING DATE: ***July 08, 2002***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
YOSSEF	KLIGER	RISHON LE'ZION, ISRAEL
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AMOTZ	NECHUSHTAN	ASERET, ISRAEL
DAVID	SPRINZAK	REHOVOT, ISRAEL

☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

QUANTITATIVE PATTERN ANALYSIS OF MOLECULES ON INTACT CELLS USING AUTOMATED SEM

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ Customer Number 24505 → *Place Customer Number Bar Code Label here*

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification *Number of Pages* 29 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 8 ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees

☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 501380

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No

☐ Yes, the name of the U.S. Government agency and the Government contract number are. ____________

Respectfully submitted,

SIGNATURE [signature]

Date 7 / 8 / 02

TYPED or PRINTED NAME DANIEL J. SWIRSKY

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Docket Number. 1143-USP

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL for FY 2002

Patent fees are *subject to* annual revision

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	JULY 8, 2002
First Named Inventor	KLIGER, YOSSEF
Examiner Name	
Group Art Unit	
Attorney Docket No.	1143-USP

METHOD OF PAYMENT

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	208	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80.00

SUBTOTAL(1) ($) 80.00

2. EXTRA CLAIM FEES

	Extra Claims		Fee from below	Fee Paid
Total Claims	-20- =	X		
Independent Claims	- 3- =	X		
Multiple Dependent				

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater, For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for ex parte reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
11 3	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1.1 7(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

SUBMITTED BY				Complete (if applicable)	
Name (Print/Type)	DANIEL J. SWIRSKY	Registration No (Attorney/Agent)	45,148	Telephone	011-972-2-999-1035
Signature				Date	JULY 8, 2002

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Provisional Patent Application

Title: Quantitative Pattern Analysis of Molecules On Intact Cells Using Automated SEM

Inventors:

Yossef Kliger, an Israeli citizen, of Rishon Le'Zion.

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David Sprinzak, an Israeli citizen, of Rehovot.

FIELD OF THE INVENTION

The present invention discloses a device and methods for analyzing the pattern and the spatial distribution of biological molecules in intact cells at cellular and subcellular levels, with the resolution of electron microscopy combined with the throughput and ease of sample preparation of light microscopy. The invention specifically enables analyzing the distribution of proteins on the cell surface by means of scanning electron microscopy, more particularly providing means enabling automated scanning electron microscopy using gold as well as fluorescence labeling, wherein the samples may be dry or wet, live or fixed.

BACKGROUND OF THE INVENTION

The complex array of structure-function relationships in biochemical processes put an ongoing challenge to researchers in the area. One major limitation in such studies is the difficulty in tracking biochemical processes, qualitatively and quantitatively, within the cellular context. A specific subgroup of proteins where such an analysis can be of merit is cell-surface receptors, as this class of proteins

constitutes more than fifty percent of the currently known drug targets.

Information such as protein translocation, distribution of membrane proteins and distribution of proteins in relation to specific organelles, is important for the understanding of cellular processes as well as for examining drug action.

One specific example of such processes are the signaling pathways, which involve receptor-ligand interactions, wherein a change in the receptor configuration or in its spatial distribution on the cell surface reflects a change in the cell status. Elucidating receptor - ligand interactions may enlighten many processes, including pathological developments leading to a disease, and may therefore also have a crucial diagnostic value. However, most existing techniques used in this research area are indirect, measuring the result coming of the receptor change and not the change itself. In particular, changes in the spatial distribution of receptors such as homo and hetero dimerization are not currently assayed.

Cell examination by various electron microscopic techniques would have served as an excellent tool for the direct measurements of living cells; yet, existing techniques still lack fundamental capabilities for analyses of live or fixed wet samples. This long-needed analysis can be achieved by combining the properties of high resolution, high signal to noise ratio, the option to inspect wet samples, automation (which requires low input sample preparation and enables screening of many samples) and the ability to measure dry or wet samples without the need of careful drying and coating.

One additional limitation of presently available techniques is the tagging system: fluorescent tagging, typically scanned with a laser scanner, tends to undergo bleaching after one scanning, meaning that the fluorescent tags emit light that is below the required intensity for detection, lowering the sensitivity and suffering also

from poor signal to noise ratio and resolution. Tagging methods that enable the use of scanning electron microscopy acquire the limitations of the EM devices, including a small scanned area that, in the absence of automation, and limits the ability to have statistical analysis required in studying such events.

The present invention relates to a novel technology utilizing known principles of automatic SEM from different industries, such as the semi-conductor industry, in which Wafer-Inspection Scanning Electron Microscopy (WISEM), disclosed in US Patents 6072178, 5644132, 5502306, 4618938 and 4609809, the contents of which are incorporated herein by reference, are widely used for quality control during the manufacturing process. The use of WISEM and ESEM was previously disclosed by one of the present inventors (WO 02/14830) to inspect fixed arrays of isolated biological molecules outside their natural environment, e.g. the cell. The present invention uses these principles for analyzing the pattern and the spatial distribution of biological molecules in intact cells at cellular and subcellular levels.

The ability to scan wet samples was disclosed in co-pending International Application (WO 02/45125) by one of the present inventors, the content of both applications being incorporated herein in their entirety.

However, the ability to obtain information regarding molecular pathways and inter-molecular interactions within the context of intact cells was not taught by any of these disclosures.

Thus, there is a great need for, and it would be highly advantageous to have methods and devices that will enable automated measurements of biological molecules under natural, minimally disrupted or unperturbed environment.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods and devices for

analyzing the pattern and the spatial distribution of biological molecules that are based on single-molecule detection, show high sensitivity and are capable of measuring the monomeric, dimeric or multimeric state of the molecule of interest. It is another object of the present invention to provide the means to enable such analyses in an intact cell, in a dry as well as in a wet sample.

It is yet another object of the present invention to provide such methods for analyzing the pattern and the spatial distribution of biological molecules in an automated manner, enabling the requisite number of measurements for proper analyses.

The present invention describes technology that for the first time permits the use of automated SEM in cell biology, by combining existing automatic SEMs with liquid handling and robotics developed mainly for automated fluorescence microscopy. For this purpose the present invention describes an array of chambers comprising cavities in which the samples are grown, and the features required to allow the combination of these arrays with existing SEM equipment. In addition, the present invention describes assays for patterning of molecules on a biological specimen. Therefore, the present invention allows, for the first time, automated cell-based assays using SEM, capable of discerning molecular interactions at resolutions previously unattainable.

In one aspect the present invention provides a method for analyzing the pattern and spatial distribution of at least a first detectable member relative to a second detectable member in a biological specimen, the biological specimen comprising intact cells, comprising the steps of labeling at least one detectable member; loading the specimen into the specimen chamber of a scanning electron microscope; irradiating the specimen with an electron beam, observing at least one of secondary, backscattered electrons and photons emerging from said chamber, thus obtaining an

image representing at least one member of the specimen; and analyzing the image for the spatial distribution of said members by image analysis software.

In one embodiment the members are biological molecules, wherein said biological molecules may be identical, capable of changing cellular spatial distribution according to specific physiological condition either stimulated or not.

In one embodiment the first and the second detectable members are biological molecules, wherein said biological molecules may be identical, capable of creating homo-multimers, or different, capable of creating hetero-multimers.

In another embodiment, one of the detectable members is a biological structure, such as membrane domain or subcellular organelle.

In yet another embodiment, one of the detectable members is an exogenous protein like viral or bacterial.

In yet another embodiment one of the detectable members is a viral particle.

In a preferred embodiment, the biological molecules are labeled with a marker suitable for detection by SEM, exemplified by, but not restricted to scintillating beads or heavy atoms, such as gold, silver or iron.

In one preferred embodiment the diameter of the heavy metal colloids (e.g., gold), is at the range of 1-200 nm, more preferably less than 20 nm.

In yet another embodiment, multi-labeling is achieved, for example by using gold colloids of a plurality of sizes, or by using combination of gold colloids and fluorescent labels, or by using a plurality of metals that are read by the X-RAY reading apparatus of the SEM, such as by Energy Depressive Spectrum and the like.

According to one embodiment of the present invention the scanning electron microscope is a wafer inspection SEM (WISEM) typically used in the microelectronic industry. The irradiation of the specimen is carried out in such a way as to form

sufficient contrast of the electrons that are back scattered from the labeled molecules in comparison with those that are emitted/scattered from the background. Surprisingly, the electrons that are back scattered from a non-labeled, non-coated cellular surface are intense enough to enable clear imaging of the cellular surface without interfering with the image obtained for the labeled molecules.

In yet another embodiment the SEM system is an environmental scanning electron microscope (ESEM) that works at almost atmospheric pressure, thus minimizing the need to prepare the specimen for vacuum.

In yet one preferred embodiment the SEM system is a system adapted for imaging of wet samples, as disclosed in co-pending International Application (WO 02/45125) by the present authors, the content of which is herby incorporated in its entirety.

In yet another currently preferred embodiment the SEM system is furnished with automation compatible chamber that enables electron microscopy of single and multiple samples.

Any image analysis software that can automatically detect markers, such as gold colloids, may be appropriate. According to yet another aspect the image analysis of the present invention may comprise any one of: performing edge detection or image segmentation analysis algorithms to identify the colloids in each region-of-interest (ROI) and counting the colloids; counting fluorescence signals, identifying X-ray spectrum of each particle for identification by comparison to a reference spectrum, and algorithms for pattern distribution analysis, such as clustering algorithms.

The present invention further relates to a device for inspection of a specimen of biological molecules, said biological molecules associated with or present in a specimen comprising intact cells, according to the above method. The device is a

scanning electron microscope (SEM) comprising an electron source to provide an electron beam; a charged particle beam column to deliver and scan an electron beam from the electron source on the surface of the specimen; a vacuum system including a first and a second chamber in each of which pressurization can be performed independently to permit loading or unloading of a first specimen in one chamber while simultaneously inspecting a second specimen; at least one electron detector; and/or fluorescence detector; means for measuring X-ray spectrum; a continuously moving x-y stage disposed to receive the specimen and to provide at least one degree of motion to the specimen while the specimen is being scanned by the electron beam; and means for carrying out image analysis of the spatial patterning of the biological molecules of the specimen.

In one preferred embodiment, the charged particle beam column is a microcolumn.

In yet another preferred embodiment, the device for inspection of a specimen of biological molecules is a wafer inspection scanning electron microscope (WISEM), adapted for biological specimens similar to the devise described in the co-pending International Application WO 02/14830, the content of which is herein incorporated by its entirety.

In yet further preferred embodiment, the device for inspection of a specimen of biological molecules is an environmental scanning electron microscope (ESEM) that works at almost atmospheric pressure, thus minimizing the need to prepare the specimen for vacuum.

In yet one another preferred embodiment the ESEM system is a system adapted for imaging of wet samples, as disclosed in co-pending International Application (WO 02/45125) by one of the present inventors, the content of which is hereby incorporated

in its entirety.

In yet another currently preferred embodiment the ESEM system is furnished with automation compatible chamber that enables electron microscopy of single and multiple samples.

In yet another aspect, the present invention provides a method for the inspection of biological molecules on a specimen using an electron beam, the method comprising localizing the biological molecules in space and labeling them with markers; pre-aligning specimen and read specimen number; reading a recipe that contains the information to be detected; loading the specimen on X-Y-T stage (T means tilt) of an electron beam device; aligning the specimen; moving XYT stage to analysis position; positioning the electron beam on the substrate accurately by measuring the position of the substrate; scanning the specimen at low resolution to create a specimen map, while enhancing contrast; determining the regions-of-interest (ROI) spots on the map that should be scanned in a high resolution; scanning the ROIs with the electron beam as the substrate is continuously moving with at least one degree of motion in an x-y plane; detecting electrons emanating from the substrate as a result of previous step and forming an image; enhancing the image contrast; storing both modified and bare image; analyzing the ROIs; and displaying the results.

In one embodiment, the specimen analyzed by such method is a wet sample.

In yet another embodiment, the specimen analyzed by such method is a dry sample, wherein the method further comprising the step of preparing the specimen for vacuum.

The specimens are prepared for vacuum by known standard methods that include drying to the required degree, protection with a membrane and freezing to prevent out-gassing. The preparation may also include fixation and coating with a

conductive layer to prevent charge accumulation; the routine of the present invention excludes this step.

The present invention successfully addresses the unmeet need for identifying and tracking complex biochemical processes with minimal interference of the cell natural states.

The present invention is explained in greater details in the description, Figures and claims below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1 Describes an automated system for the evaluation of the spatial distribution of biological molecules, specifically receptors on the surface of cells.

FIG 2 shows a detailed description of a cell containing cassette (20) according to one embodiment of the present invention.

FIG. 3 describes the membrane partition of the cassette.

FIG. 4 displays one embodiment of the current invention.

FIG. 5 shows immuno-gold labeling of HeLa cells using anti epidermal growth factor receptor (EGFR) monoclonal antibodies.

A. Control cells

B. Cells treated with epidermal growth factor (EGF) at 4°C, to induce oligomerization without internalization, prior to labeling.

FIG. 6A is a histogram of the distribution of the distances of each label to its nearest neighbor label, in HeLa cells immuno-gold labeled with anti-EGFR monoclonal antibodies after EGF application. The results are an average of 4 independent measurements. Solid line represents the theoretical random distribution of distances to nearest neighbors using the same average density of labels.

FIG. 6B is a histogram of the distribution of the distances of each label to its nearest

neighbor label in a random Monte Carlo simulation using the same density of labels and the same number of receptors. The results are an average of 4 independent simulations.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is an object of the present invention to use scanning electron microscopy for analyzing the pattern and the spatial distribution molecules in intact cells.

It is another object of the present invention to integrate the known principles of wafer inspection scanning electron microscopy (WISEM) into the field of cell biology.

It is a further object of the present invention to expand this integration by incorporating the technique of environmental scanning electron microscopy (ESEM).

It is yet another object of the present invention to include adaptations of the SEM or ESEM, such as the possibility to image a wet sample as disclosed by one of the present inventors in the co-pending International Application WO 02/45125, and the use of automation compatible chamber as described herein below.

The present invention relates to methods and devises for analyzing the pattern and the spatial distribution of biological molecules, wherein the method and the device allows highest sensitivity of ultimately single molecule detection and yields high signal to noise ratios, while at the same time retain simple sample preparation readily applicable for high throughput screening.

As used herein, the term biological molecule includes any molecule with biological relevance that may be associated with an intact cell, either intracelhularly or extracellularly. This includes, but is not limited to: polysaccharides, small chemical molecules such as lipids, peptides, hormones and other messengers, ATP GTP etc., drugs, non proteinaceous antigens and any homo- (e.g., protein-protein as example)

and hetero- (e.g., drug-protein, DNA-RNA, DNA-protein, etc.) complexes, as well as chemically modifications and derivatizations whether naturally occurring or man made, of all these different molecules.

In one aspect the present invention provides a method for analyzing the pattern and spatial distribution of at least a first detectable member relative to a second detectable member in a biological specimen comprising intact cells, comprising the steps of labeling at least one detectable member; loading the specimen into the specimen chamber of a scanning electron microscope; irradiating the specimen with an electron beam observing at least one of secondary electron, backscattered electrons and photons emerging from said chamber, thus obtaining an image representing at least one member of the specimen; and analyzing the image for the spatial distribution of said members by image analysis software.

In one embodiment the first and the second detectable members are biological molecules. The biological molecules can be identical or different, and are capable of creating multimers; identical molecules form homo-multimers while different molecules form hetero-multimers.

In another embodiment one of the detectable members is a biological structure, for example a cell organelle, or a domain in the plasma membrane.

The methods and devises disclosed in the present invention enable to determine whether a molecule is in the state of monomer or multimer. When the biological molecule is a receptor on the membrane cell, as exemplified herein below, this method provides the means for elucidating the relationships between the cell status and a specific receptor status, the information being highly valuable for understanding and diagnosing pathological conditions.

For example, many types of cancer are characterized by changes in the

abundance or activity of cell surface receptors, which often transmit external signals into the cell. Receptors for Epidermal Growth Factor (EGF) are involved in a wide variety of human cancers. Critical parameters in the activity of cell surface receptors are the amount of receptors displayed on the surface, the clustering of these molecules to groups of dimers or other polymers (requirement for activation), and the removal of the receptors from the surface after activation. Another aspect is the prevalence of homo- and heterodimers of the different subtypes of the EGF receptor (known as erbB1 – erbB4), which cannot be addressed by standard radiometric or fluorescent assays. The high resolution and signal to noise ratio of the SEM and method disclosed by the present invention make it uniquely suitable to measure these parameters. As such receptors are prime targets for anti-cancer drugs, the methods and devices disclosed in the present invention can serve as a powerful tool for drug discovery and drug screening.

Additional examples of receptors and other proteins that play a key role in pathological disorders, thus amenable to be studied by the methods and devices of the present invention and benefit drug discovery are fibroblast growth factor receptors (FGFR) involved in growth of cartilage and bones; The glucose transporter 4 protein (GLUT4), one of the most relevant parameters of insulin activity; Cell surface molecules involved in the cell immune system, such as CD4 and CCR5 that HIV-1, has to bind to in order to infect the cell; and different receptors responsible for the interaction of cells with disease-causing bacteria or viruses.

In a preferred embodiment, the biological molecules imaged by the methods and devises of the present invention are labeled directly or indirectly with a marker suitable for detection by SEM, exemplified by, but not restricted to scintillating beads or heavy atoms. Preferably the biological molecules are indirectly tagged with a heavy

atom. The heavy atom can be any atom capable of scattering particles better than the atoms that are present in all organic molecules, such as C, H, O, as well as N, S and P. Suitable heavy atoms include gold, silver and iron, which are frequently used in electron microscopy. Methods are known to link such heavy atoms to a variety of organic molecules. For example, gold can covalently bind through an SH group which is natural to proteins and can be introduced in other molecules such as nucleic acids and polysaccharides. Other heavy atoms can be trapped in suitable chelators, which can be linked to a variety of macromolecules using methods well known in the art.

More preferably, the heavy atom is gold in colloids having a diameter in the range of 1-200 nm, most preferably having a diameter less that 20 nm.

The colloidal gold creates a high intensity back scattered electron signal, and therefore, a high image contrast.

In one embodiment, there is one label per target molecule.

In yet another embodiment, multi-labeling of more than one target molecule is achieved, for example by using gold colloids of a plurality of sizes, or by using combination of gold colloids and fluorescent labels, or by using a plurality of metals that are read by the X-ray reading apparatus of the SEM, such as by Energy Depressive Spectrum and the like.

According to one embodiment the scanning electron microscope used in the method of the present invention is a wafer inspection SEM (WISEM) typically used in the microelectronic industry. The irradiation of the specimen is carried out in such a way as to form sufficient contrast of the electrons that are back scattered from the labeled molecules in comparison with those that are emitted/scattered from the background. Surprisingly, the electrons that are back scattered from a non-labeled,

non-coated cellular surface are intense enough to enable clear imaging of the cellular surface without interfering with the image obtained for the labeled molecules.

In yet another embodiment the SEM used in the method of the present invention is an environmental scanning electron microscope (ESEM) that works at almost atmospheric pressure, thus minimizing the need to prepare the specimen for vacuum.

In yet one preferred embodiment the ESM or ESEM used in the method of the present invention is a system adapted for imaging of wet samples, as disclosed in co-pending International Application (WO 02/45125) by some of the present authors, the content of which is herby incorporated in its entirety.

In yet another currently preferred embodiment the SEM or ESEM used in the method of the present invention is furnished with automation compatible chamber that enables electron microscopy of single and multiple samples, as described in Figs. 1-4.

An automated system for the evaluation of the spatial distribution of biological molecules, specifically receptors on the surface of cells, is depicted in Figure 1. A scanning electron microscope (10) is fitted with automated stage and controls and dedicated image analysis software that control the movement of the stage. Said scanning electron microscope is fed with sample cassettes (20), through a vacuum load lock, from a cassette stack holder (30) in a vacuum compatible fashion (prior art). The cassette (20) is fed into the holder by a prior art robotic arm (40). Prior to inspection in the SEM the samples are scanned with a prior art light microscope (50). The preliminary inspection enables the registration of cells coordinates and the identification of cells and regions of interest. The process is controlled by a computer (60), which also displays the results.

A detailed description of a cells cassette (20) according to one embodiment of the present invention, is shown in Figure 2. The cells (21) are located inside an array

of cavities (22), detailed in Fig. 3 hereinbelow. The array of cavities is attached to a sealant spacer (23), typically made of silicon, which connects the array (22) to a well plate containing typically 96 wells, and means (25) to close and seal the cassette (20). The top surface of the cassette (28) is tightened to the bottom well plate (24) so that all the cavities are aligned. An array of light guides (81), preferably made of plastic, is placed inside each of the wells in (24), so that when the sample is closed, the light guides are immersed on the cell medium. The light guides placed in the scanned well (84) transmit light (photons) (92) to another light guide (82), via an index matching, vacuum resistant oil (83). Said light guide (82) is connected to a photo-multiplier via a vacuum feed-through. The electrons (91) that are back scattered from the sample are detected at the backscattered electron detector (94). A heating and temperature stabilization element is connected to the array (22), making use of the fact that the array of cavities is electrically conductive and therefore also a good conductor of heat.

The membrane partition of the cassette is shown in Figure 3. An electron transparent membrane (26), protected by a grid (27) is attached to an array of cavities (29) by attachment means (25), said attachment means are one of silicon sealant shim, UV activated glue, epoxy glue or other. The array of cavities is detailed in Fig. 4 herein below. The array of cavities is attached to the multi-well plate via sealant attachment means (23).

One embodiment of the current invention is displayed in Figure 4. The data are obtained from backscattered electrons only in a dry or ESEM mode of a prior art Scanning Electron Microscope. In this embodiment, the cassette (29) is made from a flat surface preferably made of glass or silicon; the cells are situated inside flat cavities in the substrate. Said cavities are about 2mm deep. Samples should be prepared for vacuum. This can be done is any appropriate way, such as critical point

drying, chemical drying, freeze drying, and air drying.

According to yet another aspect the image analysis of the present invention may comprise any one of: performing edge detection or image segmentation analysis algorithms to identify the colloids in each region-of-interest (ROI) and counting the colloids; counting fluorescence signals; identifying X-ray spectrum of each particle for identification by comparison to a reference spectrum, and algorithms for pattern distribution analysis, such as clustering algorithms.

The present invention further relates to a device for inspection of a specimen of biological molecules according to the above method. The device is a scanning electron microscope (SEM) comprising an electron source to provide an electron beam; a charged particle beam column to deliver and scan an electron beam from the electron source on the surface of the specimen; a vacuum system including a first and a second chamber in each of which pressurization can be performed independently to permit loading or unloading of a first specimen in one chamber while simultaneously inspecting a second specimen; at least one electron detector; a fluorescence detector; means for measuring X-ray spectrum; a continuously moving x-y stage disposed to receive the specimen and to provide at least one degree of motion to the specimen while the specimen is being scanned by the electron beam; and means for carrying out image analysis of the molecules on the specimen.

In one preferred embodiment, the charged particle beam column is a microcolumn.

In yet another preferred embodiment, the device for inspection of a specimen of biological molecules according to the above method is a wafer inspection scanning electron microscope (WISEM) adapted for biological specimens, similar to the device described in the co-pending International Application WO 02/14830, the contents of

which is herein incorporated by its entirety. According to specific features disclosed therein for use with isolated biological molecules, it is now disclosed that the patterning of EGFR, CCR5, and the co-clustering of CD4 and CCR5 can be measured.

In yet further preferred embodiment, the device for inspection of a specimen of biological molecules according to the above method is an environmental scanning electron microscope (ESEM) that works at almost atmospheric pressure, thus minimizing the need to prepare the specimen for vacuum.

In yet one another preferred embodiment the SEM or ESEM system is a system adapted for imaging of wet samples, as disclosed in co-pending International Application (WO 02/45125) by the present authors, the contents of which are hereby incorporated in their entirety. According to specific feature disclosed therein, it is now disclosed that the patterning of EGFR, CCR5, and the co-clustering of CD4 and CCR5 can be measured.

In yet another currently preferred embodiment the SEM or ESEM system is furnished with automation compatible chamber that enables electron microscopy of single and multiple samples.

In yet another aspect, the present invention provides a method for the inspection of biological molecules on a specimen using an electron beam, the method comprising localizing the biological molecules in space and labeling them with markers; pre-aligning specimen and read specimen number; reading a recipe that contains the information to be detected; loading the specimen on X-Y-T stage (T means tilt) of an electron beam device; aligning the specimen; moving XYT stage to analysis position; positioning the electron beam on the substrate accurately by measuring the position of the substrate; scanning the specimen at low resolution to create a specimen map,

while enhancing contrast; determining the regions-of-interest (ROI) spots on the map that should be scanned in a high resolution; scanning the ROIs with the electron beam as the substrate is continuously moving with at least one degree of motion in an x-y plane; detecting electrons emanating from the substrate as a result of previous step and forming an image; enhancing the image contrast; storing both modified and bare image; analyzing the ROIs; and displaying the results.

In one embodiment, the specimen analyzed by such method is a wet sample.

In yet another embodiment, the specimen analyzed by such method is a dry sample, wherein the method further comprising the step of preparing the specimen for vacuum.

The specimens are prepared for vacuum by known standard methods that include drying to the required degree, protection with a membrane and freezing to prevent out-gassing. The preparation may also include fixation and coating with a conductive layer to prevent charge accumulation; the routine of the present invention excludes this step.

The principles of the invention, of using SEM for analyzing the pattern and spatial distribution of molecules detectable in intact cell may be better understood with reference to the following non-limiting examples.

EXAMPLES

Example 1: Determining the multimer state of EGFR by the method of the present invention

It is well accepted that the multimeric state of EGFR is a crucial step in the activation of this receptor. To determine the oligomerization events occurring during EGFR activation, HeLa cells were labeled using anti-EGFR monoclonal antibodies (mAB) following starvation and growth factor (EGF) induction. A second antibody

link to gold was attached to the anti-EGFR mAB. A control sample was treated the same except for the EGF induction. As shown in Fig. 5, higher fraction of clustered receptors is observed in cells treated with EGF (Fig. 5B) compared to the untreated cells (Fig. 5A). One way for analyzing the multimer state of the EGFR is by using the matrix of the number of labeled members in a certain area. Typical results for the spatial distribution of the average number of gold particles found in 17.5 micro m^2 for the control HeLa cells and for the EGF-treated HeLa cells are shown in table 1 below:

Table 1

Treatment	No. of Monomers	No. of Dimers	No. of Trimers	No. of Tetramers
Control	44.5	7	3	0
+EGF	49	15	4	1.33

*Results are average of 4 independent experiments.

Moriki et al. (JMB (2001) 311,1011-1026), suggests that about 80% cell-surface EGFR are in pre-formed dimers. The current accepted model is that EGF induction, as specified here, leads to close to 100% of the EGFR to be in dimers or higher order multimers. Our results show increase of 20-30% in the fraction of multimeric vs. monomeric state. Therefore, our results are consistent with what was known from the literature.

Another matrix that can be used for the same purpose is the distance distribution between each first labeled member and its nearest second labeled member.

Fig. 6 illustrates the use of this matrix for the same HeLa cell treated with EGF described above. Fig. 6A and B analyze the same data with two different methods: Fig. 6A is a histogram of the distribution of the distances of each label to its nearest neighbor label; Fig. 6B is a histogram of the distribution of the distances of each label to its nearest neighbor label in a random Monte Carlo simulation using the same density of labels and the same number of receptors. Solid line represents the

theoretical random distribution of distances to nearest neighbors using the same average density of labels. The results are average of 4 independent simulations.

Example 2: Mathematical model for estimating the absolute number of multimers from the observed distribution of labeled members.

The process of labeling members of interest is not perfect, i.e. only a fraction of the members, receptors in this particular example, are labeled. This is due to the nature of the immunological labeling and our desire to keep to the minimum the non-specific labeling. Partial labeling is an experimental limitation, which can be dealt with using an appropriate mathematical model. As an example we show herein below how we can estimate, in a simple case, the absolute number of multimers (of any order) from the observed distributions of labels imaged by the SEM. This estimation provides information on the actual distribution of receptors on the cell surface even though not all receptors are labeled.

The present mathematical model describes a simple situation, wherein only homo-multimers are considered.

Assume N receptors on a certain region of the cell surface. Out of these N receptors a fraction a_1 is monomers (i.e. Na_1 receptors are monomers). A fraction a_2 is receptors which are part of dimers (i.e. Na_2 receptors are part of dimers). A fraction a_3 is receptors which are part of trimers (i.e. Na_3 receptors are part of trimers), etc:

$$a_1 + a_2 + a_3 + \ldots = 1 \qquad (1)$$

Assume that the probability to label a receptor is f (i.e. Nf of the receptors are labeled) and that the probability of labeling one receptor does not depend on whether other receptors are labeled. This assumption is not necessarily correct in real life since it is likely that one gold colloid attached to a receptor, which is part of a multimer, can interfere with the labeling of other receptors belonging to the same multimer. Nevertheless, this assumption is adopted for simplicity.

The observed data can also be grouped to singlets, doublets, triplets etc. of labeled receptors. Assume that N_1 gold colloids are in singlets, N_2 gold colloids are in doublets, N_3 gold colloids are in triplets, etc., are observed.

In the case where all receptors are labeled (f=1), the real numbers equal to the measured numbers: $N_1 = Na_1$, $N_2 = Na_2$, $N_3 = Na_3$, etc.

To summarize:

The expected total number of labeled receptors, N_t, is:

(2) $N_t = Nf$

For simplicity, we will consider first the case where trimer is the largest multimer existing in the sample. Generalization to higher multimers is straightforward.

(3) $N_t = Nf = (Na_1 + Na_2 + Na_3)f$

The probability of observing N_1 singlets (singly labeled sites) is the sum of the probability to label monomers and the probability to singly label dimers and the probability to singly label trimers. Therefore, the expected value of N_1 is:

(4) $N_1 = N[fa_1 + f(1-f)a_2 + f(1-f)^2 a_3]$

The factor $f(1-f)a_2$ represents the probability to singly label a dimer and the factor $f(1-f)^2 a_3$ is the probability to singly label a trimer. In a similar manner, the expected value of N_2 and N_3 is given by:

(5) $N_2 = N[f^2 a_2 + f^2(1-f)a_3]$

(6) $N_3 = N[f^3 a_3]$

From equations (3), (4), (5) and (6), and given the real total number of receptors, N (say from prior knowledge), we can estimate the real values of Na_1, Na_2, and Na_3, from the observed N_1, N_2 and N_3,

For convenience we denote:

$n_1=N_1/N_t$, $n_2=N_2/N_t$, $n_3=N_3/N_t$. The above equations are then rewritten (dividing by N_t on both sides of the equations (4), (5) and (6) and using eq. (3)) as:

(7) $f= N_t/N'$

(8) $n_1=a_1+(1-f)a_2+ (1-f)^2a_3$

(9) $n_2=fa_2+ f(1-f)a_3$

(10) $n_3=f^2a_3$

So, the estimated fraction of the various multimers can be calculated by using:

(11) $$a_3 = \frac{n_3}{f^2}$$

(12) $$a_2 = \frac{n_2}{f} - (1-f)\frac{n_3}{f^2}$$

(13) $$a_1 = n_1 - (1-f)\frac{n_2}{f}$$

It is important to note that a_1 does not depend on n_3 directly but only through f. This is a property that is generalized when higher order multimers are used. So in a general solution with multimers up to an order m:

(14) $$a_i = \frac{n_i}{f^{i-1}} - (1-f)\frac{n_{i+1}}{f^i}$$

for i=1,2,3,...m-1 with m the highest order multimer, and

(15) $$a_m = \frac{n_m}{f^{m-1}}$$

f can be estimated by dividing the total number of observed labels (measured) by the total number of receptors (presumably known).

The value of a_1 is specifically interesting since (1- a_1) is the fraction of receptors which participate in multimers of any size.

Since f is the unknown factor in this calculation it is important to see whether

the observed data can provide information on it. This is achieved by the limit imposed on labeling efficiency, f, due to the observed ratio between number of doublets and number of singlets.

In practice this is done by taking the limit of a_1=0. i.e. all the receptors belong to multimers. Given the observed n_1 and n_2, the minimal value of f can be obtained by solving eq. (13) when a_1=0:

$$0 = n_1 - (1-f)\frac{n_2}{f} \tag{16}$$

The solution for Eq. 16 is:

$$f = \frac{n_2}{n_1 + n_2} = \frac{N_2}{N_1 + N_2} \tag{17}$$

This limit imposes a maximal value of the number receptors, N:

$$N = \frac{(N_1 + N_2)N_t}{N_2} \tag{18}$$

Eq. (13) can be rewritten in the form:

$$a_1 = const. - \frac{n_2}{f} = const. - \frac{n_2}{N_t}N \tag{19}$$

The uncertainty on f and N (due to a lack of accurate a-priori knowledge) can be translated to an uncertainty on a_1. Hence:

$$\Delta a_1 = \left|\frac{n_2}{f^2}\right|\Delta f = \left|\frac{n_2}{N_t}\right|\Delta N \tag{20}$$

Example 3: A functional cell-based assay for selecting compounds that inhibit the entry of HIV-1 into target cells

A functional cell-based assay for selecting compounds that inhibit the entry of HIV-1 into its target cells is disclosed as an illustrative example. It is based on the knowledge that CCR5 and CD4 are the principal receptors used by HIV-1 for entry

into cells in primary infection. Affecting CCR5 expression, spatial distribution, and/or co-clustering with CD4 is believed to be a potent anti-HIV-1 treatment strategy.

The prior art does not disclose any system with sufficient quality (in terms of resolution, throughput, biological relevance and so forth) to enable evaluation of drug candidates preventing the clustering of the viral receptors at the level of molecular interactions on the cell surface. Specifically, optical microscopes, resolution of which is limited by the wavelength of light, are practically limited to resolution in the sub micron range. In contrast, the high resolution of Electron Microscopy (EM) can reveal in-depth biological information (Singer II, et al. 2001. *J Virol* 75:3779-90). However, conventional EMs are too cumbersome, since manual systems cannot be used efficiently for drug discovery. So far, optical-fluorescence microscopy and Transmission Electron Microscopy (TEM) have been used as preferred technologies for CCR5 and CD4 studies. Fluorescence microscopy is limited by resolution and thus reveals unreliable results when trying to detect patterning of membrane proteins (Singer II, et al. 2001. *J Virol* 75:3779-90), and TEM is too cumbersome a tool to be employed for screening drug candidates. Gene transfection and over expression are required to overcome the low sensitivity of fluorescence-based techniques, and drastic procedures (e.g., freeze-fracturing or embedding and sectioning) during sample preparation for TEM may bias the results and limit the ability to survey many regions on the cell. Using electron microscopy in a cell-based assay, as described in the present invention, allows screening of drugs based on mechanism of action that was not used until now. The expected results are described below.

Good drug candidates will be defined as those that meet at least one of the following criteria:

i. Decrease CCR5 expression level. The best candidates will totally eliminate CCR5

from the cell membrane. It is worth noting that as defined in the context of the present specification, "totally eliminating CCR5" means that the number of CCR5 on the cell membrane is too low to play any significant role. Optical methods, e.g. FACS, typically can detect 10,000 molecules or more on the cell surface. In these cases there is a sufficient number of CCR5 molecules to enable the biological process. These molecules are not detected by optical means.

ii. Decrease the number of CCR5 in a microcluster

iii. Increase the distance between CCR5 molecules in a microcluster

Increase the distance between CCR5 microclusters and CD4 microclusters (According to one possible theoretical mechanism HIV-1 requires that CD4 and CCR5 will be close to each other, since the same gp120/gp41 trimer must associate with both of them)

Good drug candidates will not:

1. Influence the number of cells. This can be monitored by evaluation of other essential molecules.

2. Influence the level of CD4 on the cell membrane. This can be monitored by evaluation of other essential molecules.

3. Influence any characteristics of CD4 spatial distribution (cluster formation, number of molecules in a cluster, distances between molecules in a cluster, distances between clusters). This can be monitored by evaluation of other essential molecules.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and,

therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever chemical structure, or whatever function, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same functions can be used; and it is intended that such expressions be given their broadest interpretation.

CLAIMS

1. A method for analyzing the pattern and spatial distribution of at least a first detectable member relative to a second detectable member in a biological specimen, the biological specimen comprising intact cells, comprising the steps of: labeling at least one detectable member; loading the specimen into the specimen chamber of a scanning electron microscope; irradiating the specimen with an electron beam; observing at least one of secondary and backscattered electrons emerging from said sample, thus obtaining an image representing at least one member of the specimen; and analyzing the image for the spatial distribution of said members by image analysis software.
2. The method of claim 1, wherein the first and the second detectable members are biological molecules.
3. The method of claim 2, wherein the biological molecules are identical
4. The method of claim 2, wherein the biological molecules are different, then the labels are different
5. The method of claim 1, wherein at least one of the detectable members is a biological structure.
6. The method of claim 1, wherein the biological molecules are labeled with a marker suitable for detection by SEM, selected from the group comprising of scintillating beads or heavy metals, fluorescent probes, fluorescent proteins, X-ray emitting probes
7. The method of claim 6, wherein the biological molecules are labeled with gold.
8. The method of claim 7, wherein the biological molecules are labeled with gold colloids with a diameter at the range of 1-200 nm.

9. The method of claim 8, wherein the biological molecules are labeled with gold colloids with a diameter of less than 20 nm.

10. The method of claim 6, wherein different biological molecules are labeled with different markers.

11. The method of claim 1, wherein the scanning electron microscope is a wafer inspection scanning electron microscope (WISEM).

12. The method of claim 1, wherein the scanning electron microscope is an environmental scanning electron microscope (ESEM).

13. The method of claim 1, wherein the scanning electron microscope is adapted for scanning wet samples.

14. The method of claim 1, wherein the scanning electron microscope is furnished with automation compatible chamber enabling electron microscopy of single and multiple samples.

15. The method of claim 1 wherein the image analysis is performed by a method selected from the group consisting of performing edge detection algorithm or image segmentation analysis to identify the colloids in each region of interest, counting fluorescence signals or identifying X-ray spectrum of the particles in comparison with a reference background.

16. A scanning electron microscope comprising an electron source to provide an electron beam; an electron beam column to deliver and scan an electron beam from the electron source on the surface of a specimen, the specimen comprising intact cells in single or multiple cell chamber; a vacuum system including a first and a second chamber in each of which pressurization can be performed independently to permit loading or unloading of a first specimen in one chamber while simultaneously inspecting a second specimen; at least

one electron detector; a continuously moving x-y stage disposed to receive the specimen and to provide at least one degree of motion to the specimen while the specimen is being scanned by the electron beam; and means for carrying out image analysis of spatial patterning of molecules of the specimen.

17. The scanning electron microscope of claim 16 adapted for use with wet samples.

18. A method for the inspection of biological molecules on a specimen, the specimen comprising intact cells, using an electron beam, the method comprising localizing the biological molecules in space and labeling them with markers; pre-aligning specimen and read specimen number; reading a recipe that contains the information to be detected; loading the specimen on X-Y-T stage (T means tilt) of an electron beam device; aligning the specimen; moving XYT stage to analysis position; positioning the electron beam on the substrate accurately by measuring the position of the substrate; scanning the specimen at low resolution to create a specimen map, while enhancing contrast; determining the regions-of-interest (ROI) spots on the map that should be scanned in a high resolution; scanning the ROIs with the electron beam as the substrate is continuously moving with at least one degree of motion in an x-y plane; detecting electrons emanating from the substrate as a result of previous step and forming an image; enhancing the image contrast; storing both modified and bare image; analyzing the ROIs; and displaying the results.

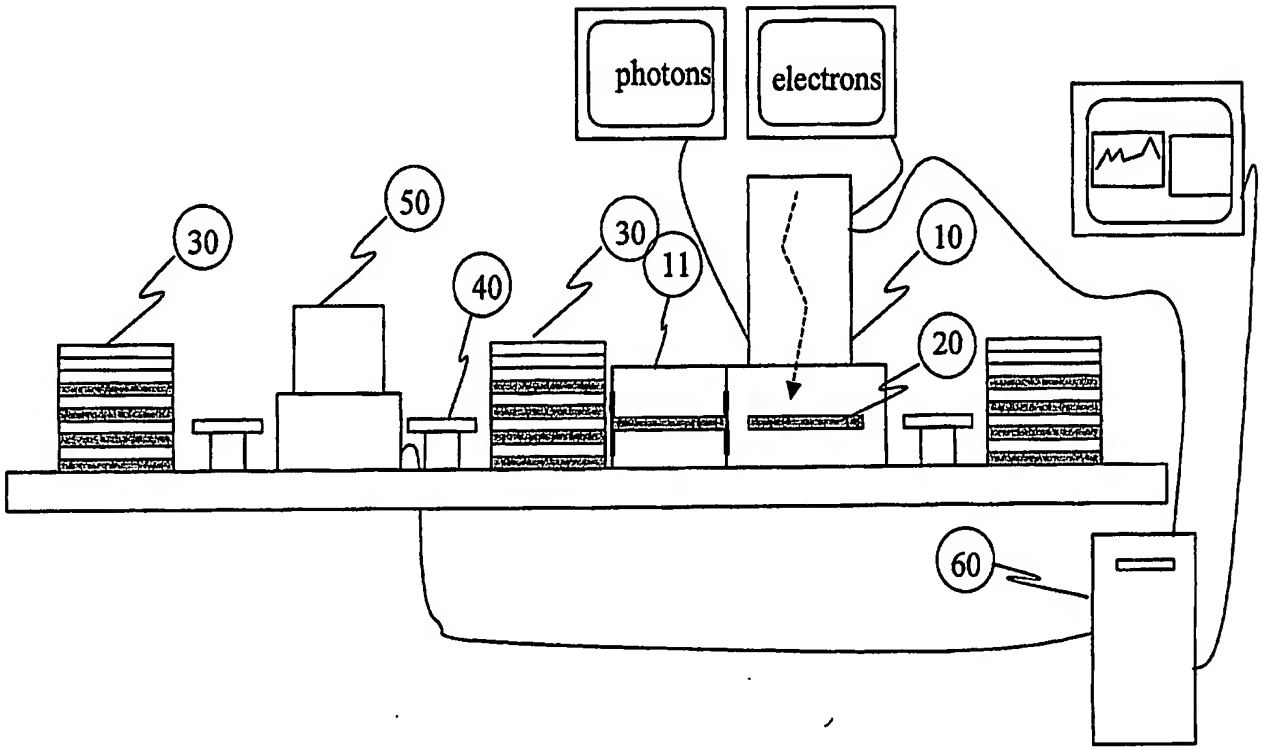


Figure 1

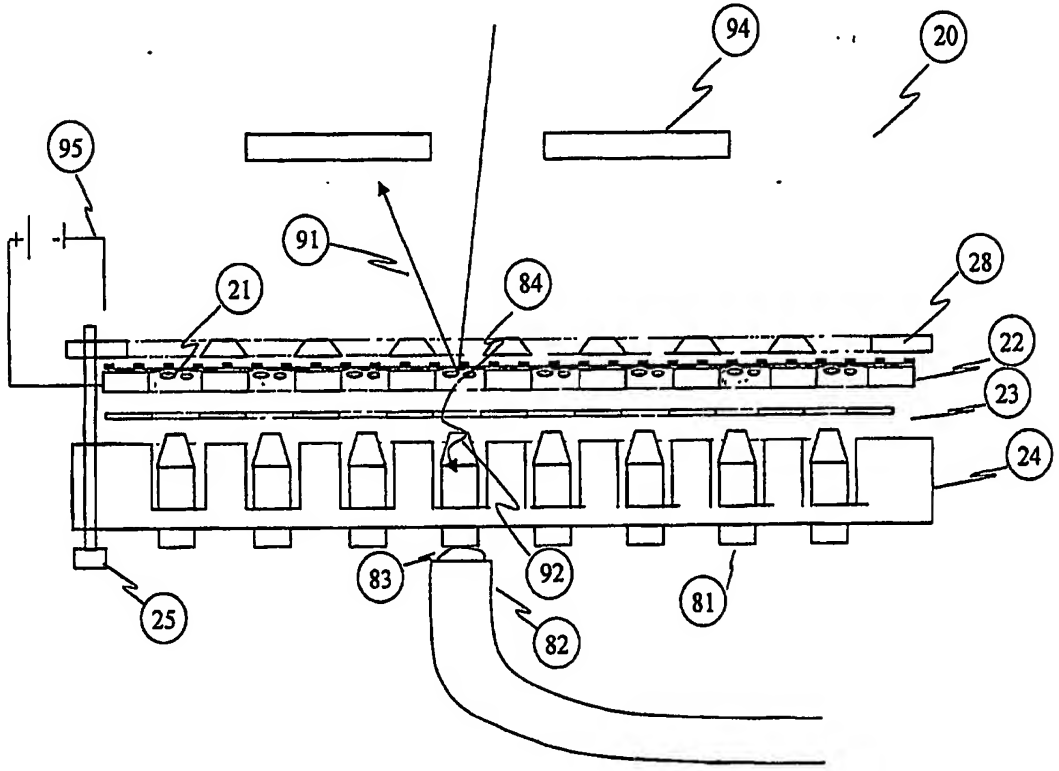


Figure 2

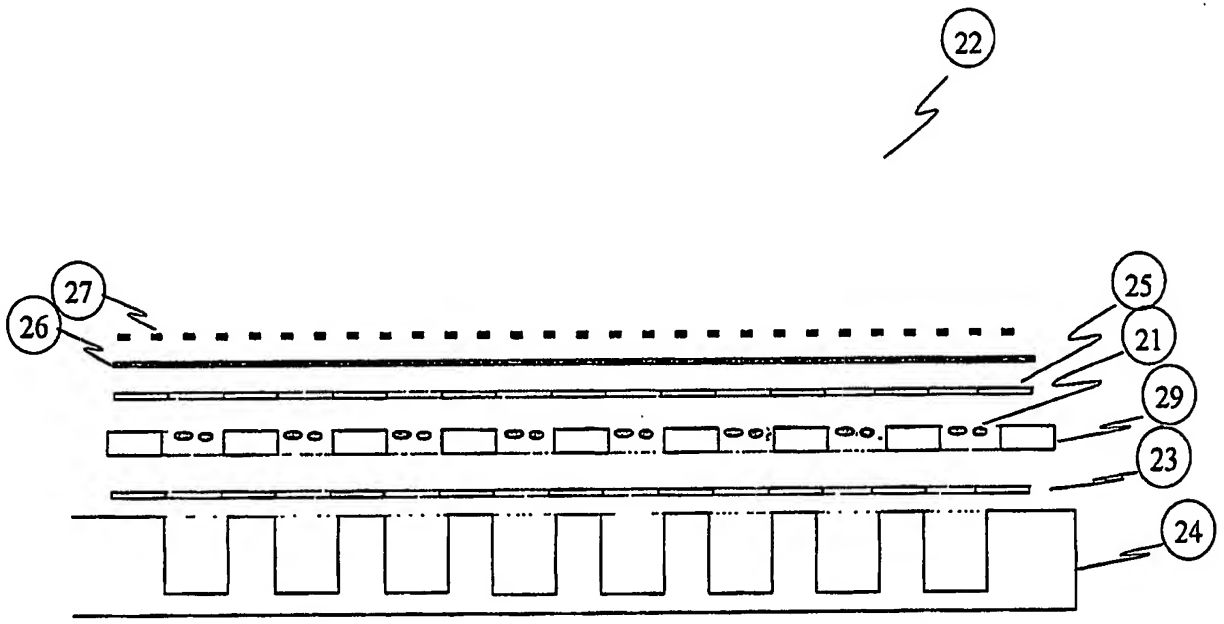


Figure 3

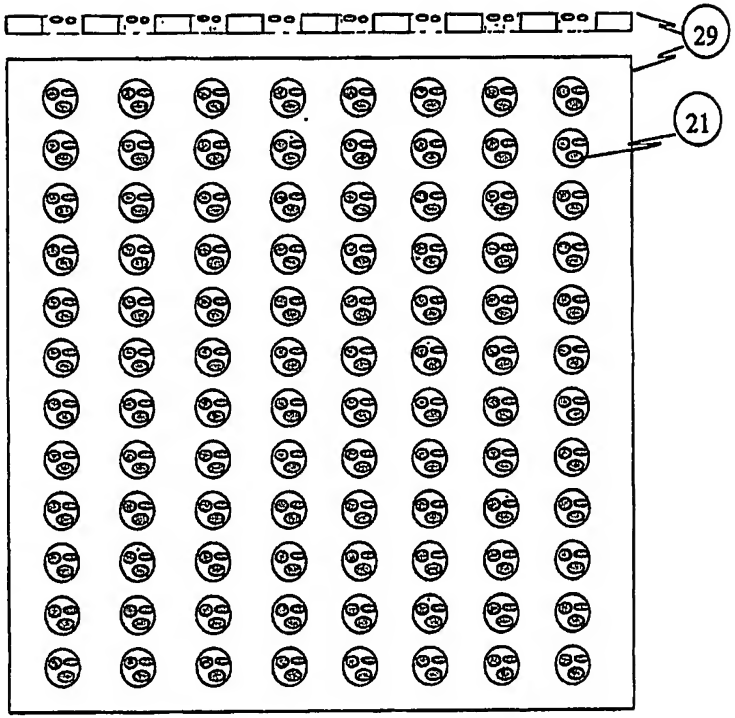


Figure 4

Figure 5A

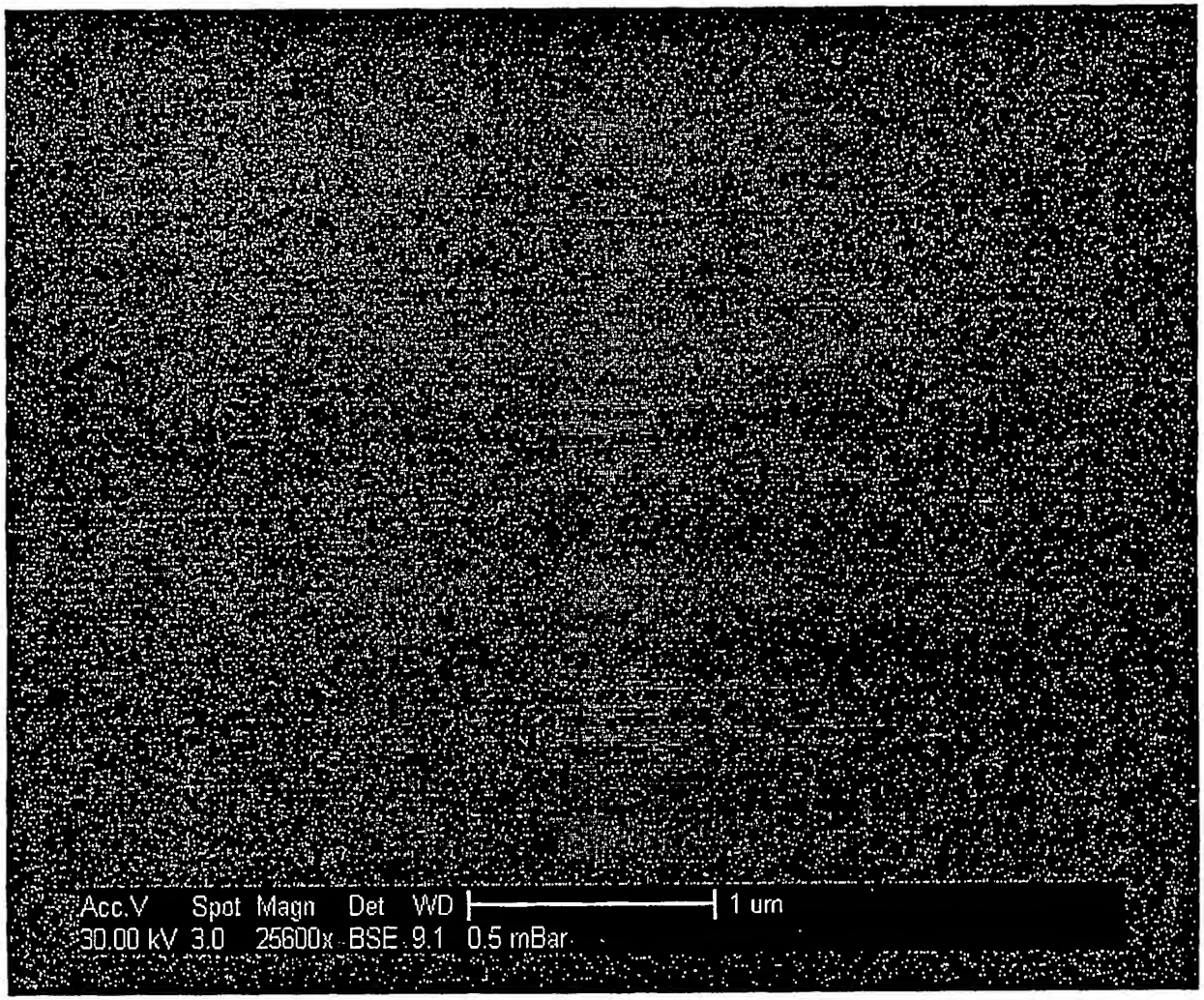
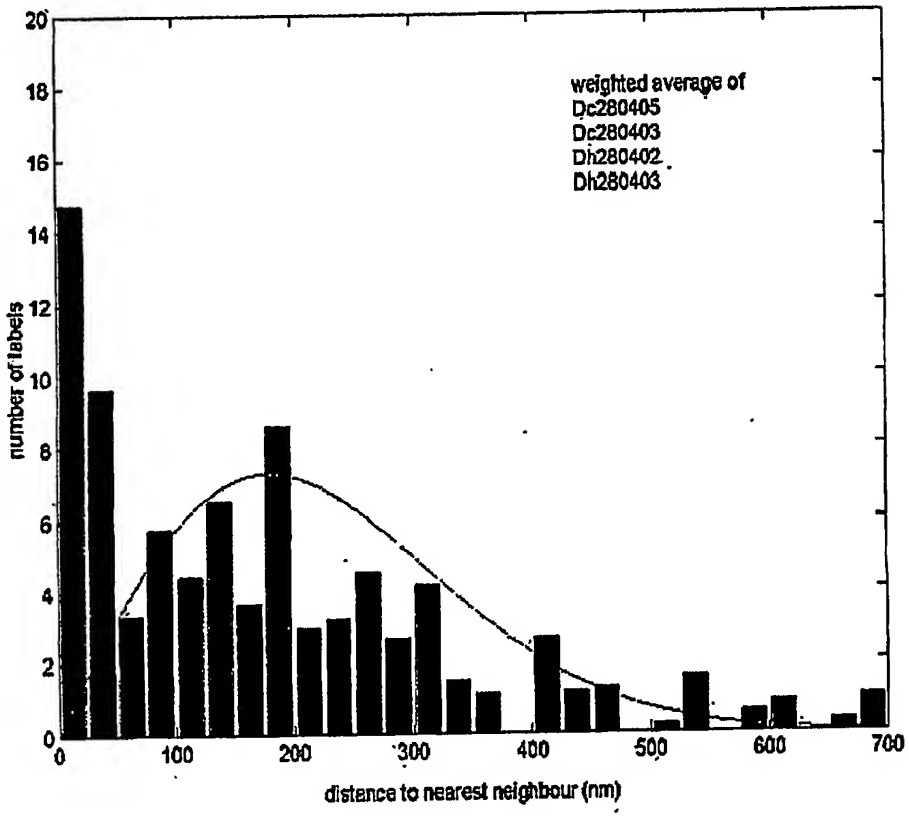


Figure 5B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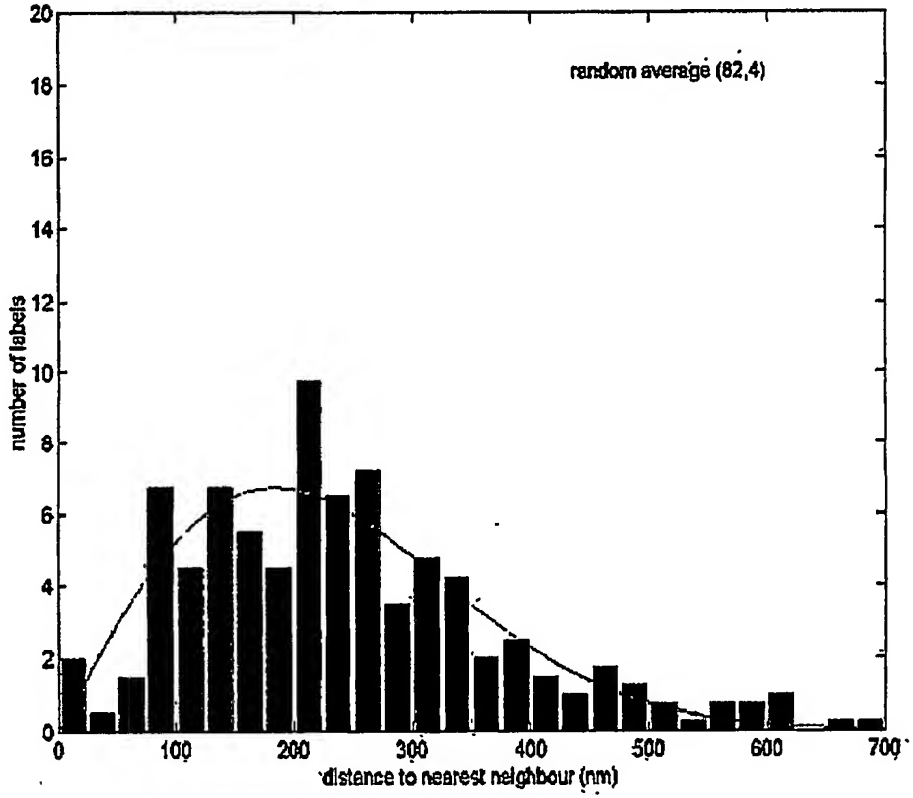
Dc280405
Dc280403
Dh280402
Dh280403
number of labels
distance to nearest neighbour (nm)
0
2
4
6
8
10
12
14
16
18
20
0
100
200
300
400
500
600
700

Figure 6A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number of labels
distance to nearest neighbour (nm)
0
2
4
6
8
10
12
14
16
18
20
0
100
200
300
400
500
600
700

Figure 6B